Ingestion of bacteria overproducing DnaK attenuates *Vibrio* infection of *Artemia franciscana* larvae

Yeong Yik Sung · Till Dhaene · Tom Defoirdt ·
Nico Boon · Thomas H. MacRae · Patrick Sorgeloos ·
Peter Bossier

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Abstract Feeding of bacterially encapsulated heat shock proteins (Hsps) to invertebrates is a novel way to limit *Vibrio* infection. As an example, ingestion of *Escherichia coli* overproducing prokaryotic Hsps significantly improves survival of gnotobiotically cultured *Artemia* larvae upon challenge with pathogenic *Vibrio campbellii*. The relationship between Hsp accumulation and enhanced resistance to infection may involve DnaK, the prokaryotic equivalent to Hsp70, a major molecular chaperone in eukaryotic cells. In support of this proposal, heat-stressed bacterial strains LVS 2 (*Bacillus* sp.), LVS 3 (*Aeromonas hydrophila*), LVS

8 (*Vibrio* sp.), GR 8 (*Cytophaga* sp.), and GR 10 (*Roseobacter* sp.) were shown in this work to be more effective than nonheated bacteria in protecting gnotobiotic *Artemia* larvae against *V. campbellii* challenge. Immunoprobings of Western blots and quantification by enzyme-linked immunosorbent assay revealed that the amount of DnaK in bacteria and their ability to enhance larval resistance to infection by *V. campbellii* are correlated. Although the function of DnaK is uncertain, it may improve tolerance to *V. campbellii* via immune stimulation, a possibility of significance from a fundamental perspective and also because it could be applied in aquaculture, a major method of food production.

Y. Y. Sung
Department of Fisheries and Aquaculture,
Faculty of Agrotechnology and Food Science,
University Malaysia Terengganu,
21030 Kuala Terengganu, Malaysia

Y. Y. Sung (✉)
Institute of Tropical Aquaculture (AQUATROP),
University Malaysia Terengganu,
21030 Kuala Terengganu, Malaysia
e-mail: yeong@umt.edu.my
URL: <http://www.umt.edu.my>

T. Dhaene · T. Defoirdt · P. Sorgeloos · P. Bossier
Laboratory of Aquaculture and Artemia Reference Center,
Ghent University,
Rozier 44,
9000 Ghent, Belgium

N. Boon
Laboratory of Microbial Ecology and Technology (LabMET),
Ghent University,
Coupure Links 653,
9000 Ghent, Belgium

T. H. MacRae
Department of Biology, Dalhousie University,
Halifax, NS B3H 4 J1, Canada

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Introduction

The brine shrimp *Artemia*, found worldwide in extreme environments, is used as a model organism for the study of stress response (Clegg et al. 2000a, b; Frankenberg et al. 2000; MacRae 2003), aquatic diseases (Verschuere et al. 2000; Soto-Rodriguez et al. 2003; Marques et al. 2005; Sung et al. 2007), feed quality (Marques et al. 2004a, b), and probiont characteristics (Marques et al. 2005, 2006a). As one example, a recently established gnotobiotic system, where larvae are hatched, grown in axenic conditions, and exposed to known bacteria, has facilitated research on host–microbe interactions, particularly as they relate to *Artemia* larvae (Marques et al. 2006b). Among the topics investigated were the contribution of bacteria toward immune stimulation in *Artemia*, the role of bacteria as a food source for this crustacean, and the influence of bacteria on the

nutritional properties of *Artemia* used as feed in aquaculture (Yasuda and Taga 1980; Hessen and Andersen 1990; Gorospe et al. 1996; Marques et al. 2005).

Physiological stress promotes synthesis of intracellular heat shock proteins (Hsps), often referred to as stress proteins or molecular chaperones, and they influence the synthesis, structure, localization, and function of other cell proteins (Parsell and Lindquist 1993). Members of the Hsp70 family, a prominent group of molecular chaperones, are robustly induced when cells experience temperature variation, oxygen deprivation, nutritional deficiency, and infection (Morimoto 1998; Feder and Hofmann 1999). Hsp70 occurs in all types of eukaryotic organisms and it has approximately 60% similarity to DnaK, the prokaryotic Hsp70 equivalent (Pockley 2003). As an unexpected finding, extracellular Hsp70, in combination with other Hsps, is thought to modulate peptide positioning on cell surfaces, thus enhancing immune recognition of aberrant cells (Athman and Philpott 2004; Johnson and Fleshner 2006). Of interest in this regard is research on economically important organisms used in aquaculture such as *Penaeus monodon* (de la Vega et al. 2006) and *Artemia franciscana* (Sung et al. 2007, 2008, 2009), where Hsp70 is implicated in protection against pathogenic microbes, perhaps by stimulation of the immune response.

Aquaculture, an important source of human nutrition, is the fastest-growing food production sector in the world, increasing about 8.8% annually during the past decade and significantly supplementing capture fisheries. With this growth is the expanding use of live food such as *Artemia* larvae. High nutritional properties, including those contributed by lipids and unsaturated fatty acids, make newly hatched *Artemia* larvae particularly suitable as a major starter diet in the rearing of farmed finfish and crustacean larvae. However, this advance necessitates rearing *Artemia* larvae under conditions where infection by bacteria and other pathogens must be controlled. In relation to this requirement, endogenous Hsp70 (Sung et al. 2007, 2008) and exogenously supplied DnaK synthesized in transformed *Escherichia coli* (Sung et al. 2009) were shown to

protect *Artemia* larvae from *Vibrio* infection, perhaps by immune stimulation. The objective of the present study was, therefore, to substantiate the observations that bacteria overproducing DnaK protect *Artemia* larvae from *Vibrio* infection.

Materials and methods

Induction of bacterial Hsp synthesis

Bacillus sp. (LVS 2), *Aeromonas hydrophila* (LVS 3), *Vibrio* sp. (LVS 8), *Cytophaga* sp. (GR 8), *Roseobacter* sp. (GR 10), and *Vibrio campbellii* strain LMG 21363 (Table 1) were stored in 40% glycerol at -80°C . Bacteria were grown at 28°C for 48 h on marine agar and then to log phase in marine broth 2216 (Difco Laboratories, Detroit, MI, USA) by overnight incubation at 28°C . Hsp synthesis was induced in bacteria, with the exception of *V. campbellii*, by a 30-min exposure to 38°C at a Δt of $4^{\circ}\text{C}/\text{min}$ in a preheated water bath. The bacterial strains were then transferred individually to sterile tubes, centrifuged at $2,200\times g$ for 15 min at 28°C , and suspended in filtered autoclaved sea water, used throughout the study. The turbidity of bacterial cultures was determined spectrophotometrically at 550 nm and these values were used to determine bacterial numbers according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells per milliliter.

Vibrio challenge of *Artemia* larvae

Axenic *Artemia* larvae were obtained by cyst decapsulation (Sorgeloos et al. 1986) followed by incubation at 28°C for 24 h in sea water (Sung et al. 2008). Larvae developing within the next 4–6 h, and thus with the ability to ingest bacteria, were harvested and incubated for 6 h with either heat-shocked or nonstressed bacteria at approximately 1×10^7 cells per milliliter. Challenge tests were then performed

Table 1 Bacterial strains

Bacteria	Strain	References
<i>Bacillus</i> sp.	LVS 2	Verschuere et al. (1999, 2000); Marques et al. (2005)
<i>Aeromonas hydrophila</i>	LVS 3	Verschuere et al. (1999, 2000); Marques et al. (2005)
<i>Vibrio</i> sp.	LVS 8	Verschuere et al. (1999, 2000); Marques et al. (2005)
<i>Cytophaga</i> sp.	GR 8	Marques et al. (2005)
<i>Roseobacter</i> sp.	GR 10	Marques et al. (2005)
<i>Vibrio campbellii</i>	LMG 21363	Soto-Rodriguez et al. (2003)

by adding pathogenic *V. campbellii* at 1×10^7 cells per milliliter and incubating with constant agitation and light for 36 h prior to recovery of swimming larvae, fixation in Lugol's solution, and counting. Survival percentage was calculated as $N_t \times 100/N_o$ where N_t and N_o are the final and initial numbers of live larvae, respectively. The increase in survival conferred by each bacterial strain upon heating was calculated as $(S_f - S_i) \times 100/S_i$ where S_f and S_i are the percentage survival of *Artemia*-fed heated and nonheated bacteria, respectively. Axenity was confirmed by plating 100 μ L of culture medium containing *Artemia*, but receiving no bacteria, on marine agar and incubating for 5 days at 28°C (Marques et al. 2004a). Results were discarded if contamination was found.

Nutritional value of bacteria

The nutritional value of bacterial strains (Table 1) was determined by a single feeding of 1×10^7 bacteria per milliliter to axenically cultured *Artemia*. Viability, as survival percentage, was determined after 2 days as previously described. Fixed nauplii were measured by using a dissecting microscope equipped with a drawing mirror, a digital plan measure, and *Artemia* 1.0® software (courtesy of Marnix Van Domme).

Statistics

Larval survival (in percent) in challenge and nutritional tests were arcsine-transformed to satisfy normality and homoscedasticity requirements while length measurements were either logarithmic- or square root-transformed as necessary. Differences in larval survival and lengths were investigated by performing analysis of variances and Tukey's multiple comparisons range using the statistical analysis software SPSS® version 11.5 for Windows®.

Immunodetection of Hsps

Protein extraction was as described (Sung et al. 2009). Bacteria were homogenized in the presence of 0.1 mm diameter glass beads in ice-cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4 , 40 mM HEPES, pH 7.4) (Clegg et al. 2000a) containing protease inhibitors (catalog no. P8465, Sigma-Aldrich, USA) at the highest recommended level. Protein concentrations were determined by the Bradford method and equal amounts of protein were loaded in each gel lane.

Bacterial proteins resolved in 10% sodium dodecyl sulfate polyacrylamide gels (Laemmli 1970) were either stained with Coomassie Biosafe (BioRad™ Laboratories, USA) or transferred to polyvinylidene fluoride membranes (BioRad Immun-Blot™ PVDF, USA) before incubating for

60 min with blocking buffer (phosphate-buffered saline containing 0.2% [v/v] Tween-20 and 5% [w/v] bovine serum albumin). Membranes were incubated for 1 h at room temperature with rabbit polyclonal antibody raised against the ATPase domain of *E. coli* DnaK (Bucca et al. 2000), a generous gift from Dr. Bernd Bukau, ZMBH, Germany. Subsequent to washing, the membrane was incubated with horseradish peroxidase-conjugated goat antirabbit IgG (Gentaur BVBA, Belgium) for 1 h, washed, and exposed to 0.7 mM diaminobenzidine tetrahydrochloride dihydrate and 0.01% (v/v) H_2O_2 in 0.1 M Tris-HCl (pH 7.6) to detect antibody-reactive proteins. The 70-kDa protein detected with this antibody in various bacterial strains is subsequently referred to as DnaK.

DnaK quantification by ELISA

DnaK was quantified with the Sensiflex™ ELISA Development Kit (Molecular Probes, Invitrogen, USA) following the manufacturer's instructions. Approximately 50 μ g of bacterial protein in 100 μ L of sodium bicarbonate buffer, pH 9.3, was added to each well of a 96-well round-bottom polystyrene plate (Nunc-Immunoplate Maxisorp, Denmark) and incubated at 4°C overnight. Plates were washed three times with phosphate-buffered saline containing 0.1% Tween-20 and blocked 4 h at 28°C with phosphate-buffered saline containing 5% bovine serum albumin. One hundred microliters of rabbit polyclonal antibody raised against *E. coli* DnaK was added to each well, and plates were incubated for 30 min at 37°C prior to washing. Goat antirabbit IgG (H+L) with β -lactamase TEM-1 conjugate was then added, and after 30 min, at 28°C, the plates were washed. Detection of antibody reactivity was by incubation for 30 min with 100 μ L of 0.9 mM Fluorocilin™ Green reagent. Fluorescence intensity was determined at 495 nm excitation and 525 nm emission in a Tecan® Infinite M200 ELISA microplate reader. A standard curve, constructed by use of recombinant DnaK, was used to convert sample absorbance to DnaK content with values expressed as *E. coli* equivalent. All experiments were done in duplicate.

Results

Heating of bacteria enhances their ability to protect *Artemia* larvae against *V. campbellii*

Feeding *Artemia* larvae heat-shocked as opposed to non-heated bacteria significantly ($p < 0.05$) increased survival upon exposure to *V. campbellii* (Fig. 1). Strain LVS 8, although conferring the lowest protection on *Artemia* larvae whether or not bacteria were heated, nonetheless, greatly improved protection when heat-shocked bacteria were

Fig. 1 Survival of *Artemia* larvae after challenge with *V. campbellii*. Larvae were fed different bacterial strains at 1×10^7 colony-forming units per milliliter prior to challenge tests. Experimental replicates are labeled A and B, and the results are presented as averages of three determinations with standard deviations (mean $\pm$ SD). CTR nonheated bacteria, HS heated bacteria. Values in the same column for each experiment showing the same superscript letter are not significantly different ($p > 0.05$). The asterisk indicates significant differences in larval survival between control and heat shock treatments

employed. In contrast, GR 10, which gave the best protection of any strain prior to heat shock, provided the least enhancement of larval protection upon heating. Increases in protection granted by heating strain LVS 3 were between that bestowed by heat shocking LVS 8 and GR 10, whereas the effects of LVS 2 and GR 8 were variable, although both provided significantly increased protection against *Vibrio* infection upon heating (Table 2).

Heat shock increased DnaK production by bacteria

Coomassie stained gels containing protein extracts from heated and nonheated bacteria of single strains were similar to one another, although LVS 2 exhibited a slight increase in a 70-kDa polypeptide upon heating (Fig. 2a). Immunoprobings of Western blots with an antibody to DnaK revealed a single reactive polypeptide of approximately 70 kDa in all strains, except LVS2 where two 70 kDa polypeptides were detected. Staining intensity of the antibody-reactive 70-kDa polypeptides was greater in protein extracts from heat-shocked versus nonheated bacteria (Fig. 2b).

Table 2 Feeding with heat-stressed bacteria increases *Artemia* resistance to *V. campbellii*

Bacteria strains	A Increase in survival (%)	B Increase in survival (%)
LVS 2	50 $\pm$ 7	139 $\pm$ 10
LVS 3	79 $\pm$ 13	59 $\pm$ 6
LVS 8	105 $\pm$ 11	116 $\pm$ 5
GR 8	47 $\pm$ 2	109 $\pm$ 5
GR 10	41 $\pm$ 4	42 $\pm$ 1

Artemia larvae were incubated in replicate experiments A and B with nonheated and heat-stressed bacteria at 1×10^7 cells per milliliter for 6 h prior to challenge with *V. campbellii* and then the number of surviving larvae were determined. The results are presented as the average of three determinations with standard deviations (mean $\pm$ SD)

Additionally, quantification by enzyme-linked immunosorbent assay (ELISA) demonstrated that DnaK increased from 2.0- to 2.3-fold in heated bacteria (Fig. 3). The higher amounts of DnaK in heated as opposed to nonstressed bacteria correlated with enhanced ability to promote survival of *Artemia* larvae.

Larval resistance to *Vibrio* infection is not determined by the nutritional value of bacteria

When *Artemia* were starved, mortality was high, even in the absence of *Vibrio* exposure, with only $9 \pm 5\%$ and $13 \pm$

Fig. 2 Heat shock stimulates bacterial DnaK synthesis. Bacterial protein extracts were electrophoresed in sodium dodecyl sulfate polyacrylamide gels and either stained with Coomassie Biosafe (a) or blotted to polyvinylidene fluoride membranes and incubated with antibody to DnaK (b). Approximately 50 μ g of protein was loaded in each lane; CTR nonheated bacteria, HS heated bacteria. Molecular mass standards in kilodaltons are on the left. Arrow 70-kDa polypeptide

Fig. 3 Quantification of bacterial DnaK. The amount of DnaK was determined in nonheated (Non-HS) and heated (HS) bacteria by ELISA and presented as the mean $\pm$ SD. The fold increase in DnaK upon heating is shown above the bars. The asterisk indicates significant difference in DnaK levels between nonheated and heated bacteria

5% of larvae alive in replicate experiments after 48 h. Conversely, incubating larvae with any of the bacterial strains employed in this study, except *V. campbellii*, significantly increased the number of live larvae ($p < 0.05$) (Table 3). Bacterial strains varied in nutritional quality. In comparison to LVS 8, feeding with both LVS 2 and LVS 3 substantially increased viable larvae, while moderate but insignificant increases were obtained with GR 8 and GR 10 ($p < 0.05$). Differences in the nutritional values of heated versus nonheated bacteria were insignificant ($p > 0.05$), except for LVS 8 and GR 8 in experiment A which gave slight but significant increases in viable larvae. In no case was the difference in nutritional value great enough to account for the enhanced survival conferred on larvae by feeding with heated versus nonheated bacteria. Regardless of heat exposure, the average length of *Artemia* larvae, which also acted as an index of bacterial nutritional value, was highest when larvae were fed strain LVS 2 and lowest when LVS 8 was employed. Generally, length variation was

insignificant when comparing results obtained by feeding larvae with LVS 2 and other bacterial strains ($p > 0.05$), although some differences were apparent (Table 3). Incubation with only *V. campbellii* killed all larvae.

Discussion

Feeding with bacteria, apart from virulent strains such as *V. campbellii* (Soltanian et al. 2007; Sung et al. 2008), *V. proteolyticus* (Marques et al. 2006a; Sung et al. 2007), and *V. harveyi* (Soto-Rodriguez et al. 2003; Defoirdt et al. 2006), enhances *Artemia* resistance against disease and infection. As one example, colonization of cultures with nine different live bacterial strains protected *Artemia* juveniles against *V. proteolyticus* CW8T2 (Verschuere et al. 1999). Moreover, dead bacteria contribute similar beneficial effects, suggesting as one possibility that enhanced tolerance of *Artemia* to virulent bacteria occurs through stimulation of the nonspecific or innate immune response (Verschuere et al. 2000; Marques et al. 2006a).

In this study, feeding gnotobiotic *Artemia* with heat-shocked bacteria significantly increased larval resistance against the detrimental effects of *V. campbellii*. Additionally, immunoprobings of Western blots and protein quantification by ELISA demonstrated that enhanced protection is associated with increased DnaK accumulation in bacteria used as feed. Previously, feeding with *E. coli* overexpressing DnaK, produced under the control of either its own promoter or a heterologous arabinose-inducible promoter, boosted *Artemia* survival approximately threefold (Sung et al. 2009). The present study extends these observations, demonstrating clearly that DnaK-producing bacteria other than *E. coli* confer protection upon *Artemia* larvae.

Table 3 Nutritional value of bacteria

Bacteria strains	A				B			
	CTR		Heat shock		CTR		Heat shock	
	Live larvae (%)	Individual length (mm)	Live larvae (%)	Individual length (mm)	Live larvae (%)	Individual length (mm)	Live larvae (%)	Individual length (mm)
LVS 2	89 $\pm$ 6a	1.3 $\pm$ 0.1a	82 $\pm$ 7a	1.3 $\pm$ 0.1a	89 $\pm$ 1a	1.2 $\pm$ 0.1a	79 $\pm$ 1a*	1.3 $\pm$ 0.1a
LVS 3	75 $\pm$ 8a	1.2 $\pm$ 0.3ab	81 $\pm$ 4a	1.1 $\pm$ 0.2a	72 $\pm$ 8b	1.1 $\pm$ 0.1ab	79 $\pm$ 3a	1.1 $\pm$ 0.2ab
LVS 8	35 $\pm$ 4b	1.0 $\pm$ 0.1b	47 $\pm$ 2b*	1.1 $\pm$ 0.1a	49 $\pm$ 8c	1.0 $\pm$ 0.1b	43 $\pm$ 1c	1.1 $\pm$ 0.2ab
GR 8	52 $\pm$ 2b	1.1 $\pm$ 0.2ab	43 $\pm$ 4b*	1.2 $\pm$ 0.1a	59 $\pm$ 8bc	1.1 $\pm$ 0.1b	59 $\pm$ 4b	1.2 $\pm$ 0.2ab
GR 10	48 $\pm$ 5b	1.1 $\pm$ 0.2ab	57 $\pm$ 7b	1.1 $\pm$ 0.2a	51 $\pm$ 6c	1.1 $\pm$ 0.1b	55 $\pm$ 3b	1.1 $\pm$ 0.1b

Artemia were incubated with (fed) bacteria at 1×10^7 cells per milliliter and live larvae were counted after 48 h. Experimental replicates are labeled A and B and results are shown as the average of three determinations with standard deviations (mean $\pm$ SD). Only 9 $\pm$ 5% and 13 $\pm$ 5%, respectively, of larvae were alive in replicates A and B when *Artemia* were not fed. Live larvae (in percent), survival after feeding; individual length (in millimeters), average length of larvae ($n=10$) 48 h after feeding; CTR, nonheated bacteria; heat shock, heated bacteria. Values in each column showing the same letter are not significantly different ($p > 0.05$). The asterisk indicates significant difference in live larvae between treatments using heated and nonheated bacteria

Moreover, the protective effects obtained by feeding many different strains of heat-shocked bacteria possessing increased DnaK suggest direct involvement of this protein.

The effects of beneficial bacteria in an aquaculture system can be explained by various mechanisms such as improvement of water quality, antagonism towards pathogens including competition for adhesion sites, enzymatic contribution to digestion in the host, and stimulation of the host immune response (Verschuere et al. 2000; Farzanfar 2006; Tinh et al. 2008). How Hsp-producing bacteria protect brine shrimp has yet to be determined, but there is compelling evidence that these proteins induce strong immunological responses in other organisms (Pockley 2003). Bacterial Hsps robustly stimulate the production of proinflammatory cytokine in human monocytes (Galdiero et al. 1997) and induce interleukin-1 secretion from macrophages (Retzlaff et al. 1994). Additionally, extracellular Hsp70 increases the production of inducible nitric oxide synthase and nitric oxide, as well as tumor necrosis factor- α , interleukin-1 β , and interleukin-6 in macrophages and neutrophils (Asea et al. 2000; Panjwani et al. 2002; Campisi and Fleshner 2003; Johnson and Fleshner 2006). Furthermore, phagocytes and granulocytes release lysozyme, reactive oxygen species, and cationic peptides upon exposure to Hsps, all of which suppress infection (Jacquier-Sarlin et al. 1994; Basu et al. 2002).

There is accumulating evidence that stimulation of the innate immune response by Hsps protects invertebrates from disease and infection. For example, buildup of Hsp70 after short-term hyperthermic stress correlates with attenuation of gill-associated virus replication in the black tiger prawn, *P. monodon* (de la Vega et al. 2006). Acting through a conserved pathway involving heat shock transcription factor-1 and the associated DAF-2/DAF-16 pathway, stress-regulated small Hsps and Hsp90 trigger immunity in the nematode *C. elegans* against *Pseudomonas aeruginosa* (Singh and Aballay 2006). Additionally, endogenous Hsp70 accretion enhances resistance of gnotobiotic *Artemia* larvae to *V. campbellii* and *V. proteolyticus* (Sung et al. 2007, 2008). In both studies, Hsps were proposed to activate the *Artemia* innate immune system, thus promoting recognition and destruction of pathogens by defensive mechanisms.

In this study, administration of heat-shocked bacteria enriched in DnaK, and perhaps other Hsps, represented an efficient strategy for biocontrol of vibriosis. This observation has applied significance in aquaculture where disease associated with luminescent vibrios causes mass mortalities of cultured organisms and economic losses worldwide (Diggles et al. 2000; Austin and Zhang 2006). Antibiotic therapy is used but this results in microbial resistance, tissue accumulation of antibiotic residues (Vadstein 1997), and immunosuppression (Hameed and Balasubramanian

2000; Smith et al. 2003), consequently posing serious threats to human health. Application of the results described in this paper has the potential to alleviate these types of problems. The current findings also comment upon the relationship between Hsps, the innate immune response, and bacterial resistance in invertebrates, all of fundamental and applied significance.

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References

- Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK (2000) HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 6:435–442. doi:10.1038/74697
- Athman R, Philpott D (2004) Innate immunity via Toll-like receptors and Nod proteins. *Curr Opin Microbiol* 7:25–32. doi:10.1016/j.mib.2003.12.013
- Austin B, Zhang XH (2006) *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Lett Appl Microbiol* 43:119–124. doi:10.1111/j.1472-765X.2006.01989.x
- Basu N, Todgham AE, Ackerman PA, Bibeau MR, Nakano K, Schulte PM, Iwama GK (2002) Heat shock protein genes and their functional significance in fish. *Gene* 295:173–183. doi:10.1016/S0378-1119(02)00687-X
- Bucca G, Brassington AME, Schönfeld HJ, Smith CP (2000) The HspR regulon of *Streptomyces coelicolor*: a role for the DnaK chaperone as a transcriptional co-repressor. *Mol Microbiol* 38:1093–1103. doi:10.1046/j.1365-2958.2000.02194.x
- Campisi J, Fleshner M (2003) The role of extracellular Hsp72 in acute stress-induced potentiation of innate immunity in physically active rats. *J Appl Physiol* 94:43–52
- Clegg JS, Jackson SA, Hoa NV, Sorgeloos P (2000a) Thermal resistance, developmental rate and heat shock proteins in *Artemia franciscana*, from San Francisco Bay and Southern Vietnam. *J Exp Mar Biol Ecol* 252:85–96. doi:10.1016/S0022-0981(00)00239-2
- Clegg JS, Jackson SA, Popov VI (2000b) Long-term anoxia in encysted embryos of the crustacean, *Artemia franciscana*: viability, ultrastructure and stress proteins. *Cell Tissue Res* 301:433–446. doi:10.1007/s004410000249
- de la Vega E, Hall MR, Degnan BM, Wilson KJ (2006) Short-term hyperthermic treatment of *Penaeus monodon* increases expression of heat shock protein 70 (HSP70) and reduces replication of gill associated virus (GAV). *Aquaculture* 253:82–90. doi:10.1016/j.aquaculture.2005.07.041
- Defoirdt T, Crab R, Wood TK, Sorgeloos P, Verstraete W, Bossier P (2006) Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemo-*

- lyticus* isolates. Appl Environ Microbiol 72:6419–6423. doi:10.1128/AEM.00753-06
- Diggles BK, Moss GA, Carson J, Anderson CD (2000) Luminous vibriosis in rock lobster *Jasus verreauxi* (Decapoda: Palinuridae) phyllosoma larvae associated with infection by *Vibrio harveyi*. Dis Aquat Org 43:127–137. doi:10.3354/dao043127
- Farzanfar A (2006) The use of probiotics in shrimp aquaculture. FEMS Immunol Med Microbiol 48:149–158. doi:10.1111/j.1574-695X.2006.00116.x
- Feder ME, Hofmann GE (1999) Heat shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282. doi:10.1146/annurev.physiol.61.1.243
- Frankenberg MM, Jackson SA, Clegg JS (2000) The heat shock response of adult *Artemia franciscana*. J Therm Biol 25:481–490. doi:10.1016/S0306-4565(00)00013-9
- Galdiero M, de l'Ero GC, Marcatili A (1997) Cytokine and adhesion molecule expression in human monocytes and endothelial cells stimulated with bacterial heat shock proteins. Infect Immun 65:699–707
- Gorospe J, Nakamura K, Abe M, Higashi S (1996) Nutritional contribution of *Pseudomonas* sp. in *Artemia* culture. Fish Sci 62:914–918
- Hameed A, Balasubramanian G (2000) Antibiotic resistance in bacteria isolated from *Artemia* nauplii and efficacy of formaldehyde to control bacterial load. Aquaculture 183:195–205. doi:10.1016/S0044-8486(99)00293-8
- Hessen D, Andersen T (1990) Bacteria as a source of phosphorous for zooplankton. Hydrobiologia 206:217–223
- Jacquier-Sarlin MR, Fuller K, Dinh-Xuan AT, Richard MJ, Polla BS (1994) Protective effects of hsp70 in inflammation. Experientia 50:1031–1038. doi:10.1007/BF01923458
- Johnson JD, Fleshner M (2006) Releasing signals, secretory pathways, and immune function of endogenous extracellular heat shock protein 72. J Leukoc Biol 79:425–434. doi:10.1189/jlb.0905523
- Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–685. doi:10.1038/227680a0
- MacRae TH (2003) Molecular chaperones, stress resistance and development in *Artemia franciscana*. Cell Dev Biol 14:251–258. doi:10.1016/j.semcdb.2003.09.019
- Marques A, Dhont J, Sorgeloos P, Bossier P (2004a) Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically grown brine shrimp *Artemia franciscana*. J Exp Mar Biol Ecol 321:115–136. doi:10.1016/j.jembe.2004.06.008
- Marques A, Francois JM, Dhont J, Bossier P, Sorgeloos P (2004b) Influence of yeast quality on performance of gnotobiotically grown *Artemia*. J Exp Mar Biol Ecol 310:247–264. doi:10.1016/j.jembe.2004.04.009
- Marques A, Dinh T, Ioakeimidis C, Huys G, Swings J, Verstraete W, Dhont J, Sorgeloos P, Bossier P (2005) Effects of bacteria on *Artemia franciscana* cultured in different gnotobiotic environments. Appl Environ Microbiol 71:4307–4317. doi:10.1128/AEM.71.8.4307-4317.2005
- Marques A, Dhont J, Sorgeloos P, Bossier P (2006a) Immunostimulatory nature of β -glucans and baker's yeast in the challenge test of *Artemia*. Fish Shellfish Immunol 20:682–692. doi:10.1016/j.fsi.2005.08.008
- Marques A, Ollevier F, Verstraete W, Sorgeloos P, Bossier P (2006b) Gnotobiotically grown aquatic animals: opportunities to investigate host–microbe interactions. J Appl Microbiol 100:903–918. doi:10.1111/j.1365-2672.2006.02961.x
- Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 12:3788–3796. doi:10.1101/gad.12.24.3788
- Panjwani NN, Popova L, Srivastava PK (2002) Heat shock proteins gp96 and hsp70 activate the release of nitric oxide by APCs. J Immunol 168:2997–3003
- Parsell DA, Lindquist S (1993) The function of heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet 27:437–496. doi:10.1146/annurev.ge.27.120193.002253
- Pockley AG (2003) Heat shock proteins as regulators of the immune response. Lancet 362:469–476. doi:10.1016/S0140-6736(03)14075-5
- Retzlaff C, Yamamoto Y, Hoffman PS, Friedman H, Klein TW (1994) Bacterial heat shock proteins directly induce cytokine mRNA and interleukin-1 secretion in macrophage cultures. Infect Immun 62:5689–5693
- Singh V, Aballay A (2006) Heat-shock transcription factor (HSF)-1 pathway required for *Caenorhabditis elegans* immunity. Proc Natl Acad Sci U S A 103:13092–13097. doi:10.1073/pnas.0604050103
- Smith VJ, Brown JH, Hauton C (2003) Immunostimulation in crustaceans: does it really protect against infection? Fish Shellfish Immunol 15:71–90. doi:10.1016/S1050-4648(02)00140-7
- Soltanian S, Dhont J, Sorgeloos P, Bossier P (2007) Influence of different yeast cell-wall mutants on performance and protection against pathogenic bacteria (*Vibrio campbellii*) in gnotobiotically-grown *Artemia*. Fish Shellfish Immunol 23:141–153. doi:10.1016/j.fsi.2006.09.013
- Sorgeloos P, Lavens P, Léger P, Tackaert W, Versichele D (1986) Manual for the culture and use of brine shrimp *Artemia* in aquaculture. Artemia Reference Center, Faculty of Agriculture, State University of Ghent, Belgium, p 318
- Soto-Rodriguez SA, Roque A, Lizarraga-Partida ML, Guerra-Flores AL, Gomez-Gil B (2003) Virulence of luminous vibrios to *Artemia franciscana* nauplii. Dis Aquat Org 53:231–240. doi:10.3354/dao053231
- Sung YY, Van Damme EJM, Sorgeloos P, Bossier P (2007) Non-lethal heat shock protects gnotobiotic *Artemia franciscana* larvae against virulent Vibrios. Fish Shellfish Immunol 22:318–326. doi:10.1016/j.fsi.2006.05.008
- Sung YY, Pineda C, MacRae TH, Sorgeloos P, Bossier P (2008) Exposure of gnotobiotic *Artemia franciscana* larvae to abiotic stress promotes heat shock protein 70 synthesis and enhances resistance to pathogenic *Vibrio campbellii*. Cell Stress Chaperones 13:59–66. doi:10.1007/s12192-008-0011-y
- Sung YY, Ashame MF, Chen SJ, MacRae TH, Sorgeloos P, Bossier P (2009) Feeding *Artemia franciscana* (Kellogg) larvae with bacterial heat shock protein, protects from *Vibrio campbellii* (Baumann) infection. J Fish Dis (in press)
- Tinh NTN, Yen VHN, Dierckens K, Sorgeloos P, Bossier P (2008) An acyl homoserine lactone-degrading microbial community improves the survival of first-feeding turbot larvae (*Scophthalmus maximus* L.). Aquaculture 285:56–62. doi:10.1016/j.aquaculture.2008.08.018
- Vadstein O (1997) The use of immunostimulation in marine larviculture: possibilities and challenges. Aquaculture 155:401–417. doi:10.1016/S0044-8486(97)00114-2
- Verschuere L, Rombaut G, Huys G, Dhont J, Sorgeloos P, Verstraete W (1999) Microbial control of the culture of *Artemia* juveniles through pre-emptive colonization by selected bacterial strains. Appl Environ Microbiol 65:2527–2533
- Verschuere L, Heang H, Criel G, Sorgeloos P, Verstraete W (2000) Selected bacterial strains protect *Artemia* sp. from pathogenic effects of *Vibrio proteolyticus* CW8T2. Appl Environ Microbiol 66:1139–1146. doi:10.1128/AEM.66.3.1139-1146.2000
- Yasuda K, Taga N (1980) A mass culture method for *Artemia salina* using bacteria as food. Mer 18:53–62